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O-GlcNAc Misregulation and Aneuploidy in Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We examined the effects of siRNA depletion of Early Mitotic Inhibitor 1(Emi1) with O-GlcNAc transferase(OGT) and O-GlcNAcase(NCOAT) on re-replication and found a modest increase in re-replication with NCOAT/Emi1 knockdown. By western analysis our knockdown has been modest, and we have further optimization of the siRNA conditions to perform. We have identified a set of microRNAs whose expression are regulated by DNA damage in breast cancer, including miR-29c, which has been shown to play a role in regulating cell survival. We have found that miR-29c expression is induced in multiple cell lines following DNA damage, and its basal expression is affected by the p53 status of the cell line. We intend to examine whether miRNAs involved in cell survival following DNA damage can act synergistically with re-replication generated by Emi1 knockdown and interference with O-GlcNAc signaling leading to malignant transformation.

15. SUBJECT TERMS
Breast Cancer, Aneuploidy, O-GlcNAc, Re-replication, Genomic Instability

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Table of Contents

Introduction:

O-GlcNAc and Emi1 in genomic instability of breast cancer.

One of the key steps in initiation and progression of breast cancer is the loss of control over DNA replication and the cell cycle that lead to genetic instability and aneuploidy. Gene amplification and transcriptional misregulation resulting from this instability represent some of the last steps in the formation of malignant neoplasias. O-linked N-acetyl glucosamine is a post-translational protein modification similar in many respects to protein phosphorylation in the number of targets that are modified and the variety of cellular pathways it modulates (1). As a byproduct of glycolosis, modification by O-GlcNAc is modulated by the availability of glucose and thus is thought to act as a nutrient sensor. Previous studies have shown that mis-regulation of O-GlcNAc can lead to faulty cell cycle regulation and aneuploidy in HeLa cells (2). Wel hypothesized that modulation of O-GlcNAc regulatory pathways and defects in control of the cell cycle and DNA repair may act synergistically in the promotion of malignant transformation. Previously, we have shown that depletion of Early Mitotic Inhibitor 1(Emi1) leads to cell cycle defects and re-replication in normal breast epithelial cells (3). In this study, we have proposed to examine whether siRNA knockdown of Emi1 along with O-GlcNAc Transferase(OGT) and O-GlcNAcase(NCOAT), enzymes that regulate O-GlcNAc modification of cellular proteins, can act synergistically to cause large scale re-replication, aneuploidy and eventually lead to malignant transformation in breast epithelial cells.

Supplemental Aim: Determining the role of microRNAs in the response to DNA damage in breast cancer.

A great deal of recent attention has been placed on the role of microRNAs in cancer, especially in regards to cell survival and proliferation. MicroRNAs are a recently discovered class of small RNA molecules that post-transcriptionally regulate the expression of a plethora of genes and cellular pathways. They play a role in the differentiation and function of numerous cell types across species, and some have been shown to dramatically affect cell survival (4,5). They have been shown to regulate both p53(6) and the estrogen receptor (7), and have the potential to contribute to the progression of breast cancer. They also represent excellent therapeutic targets as they can now be readily introduced as well as inhibited both in cells in culture as well as *in vivo* in animals or patients (8). The function and regulation of miRNAs are collectively a focus of this lab, as is the role of genomic instability in cancer, and the examination of their potential role in the response of breast cancer to DNA damage ties into the goals of this project. We intend to identify microRNAs whose expression is modulated in response to DNA damage, to identify microRNAs that may be able to enhance normal breast epithelial cells ability to cope with re-replication stress. Stable expression of a pro-survival microRNA may then allow cells undergoing re-replication by perturbation of O-GlcNAc signaling and Emi1 to continue to proliferate and undergo malignant transformation.

Body:

Task 1: Testing checkpoint activation, cell cycle effect and re-replication after co-depleting Emi1 and OGT or NCOAT

Co-depleting NCOAT and Emi1 may result in genomic instability

Our first goal is to determine if Emi1 knockdown in conjunction with misregulation of O-GlcNAc post-translational modification of proteins can act synergistically to cause re-replication. We have obtained antibodies as well as siRNA oligos against OGT, NCOAT and Emi1. We treated MCF7 and Sk-Br-3 breast cancer cells as well as MCF10a breast mammary epithelial cell lines with 20nM siRNA oligos targeting various combinations of OGT, NCOAT, Emi1 and GL2 firefly luciferase using Invitrogen Lipofectamine RNAimax transfection reagent. The cells were collected 72 hours post-transfection and analyzed by flow assisted cell sorting(FACS) to determine the cell cycle profile and whether re-replication was occurring in these cells. Cells were stained with propidium iodide for 1 hour and DNA content was analyzed by FACS. Cell cycle analysis was performed using FloJo software. In addition, whole cell lysates were collected from these samples to perform western blot to assess depletion of the proteins targeted by the siRNAs and to examine various cell cycle and checkpoint markers.

In the normal mammary epithelial cell line MCF10a we saw a modest increase in the >4N DNA content population, as well as a decrease in the G1 population, in the NCOAT/Emi1 knockdown sample as compared to the GL2/Emi1 siRNA control indicating that some re-replication may be stimulated by the co-knockdown of these proteins (Fig. 1a). We observed little cell cycle effect in MCF7 or Sk-Br-3 (Fig. 1b, 1c). This data contrasts with our previous experience in that siRNA against Emi1 has been shown to generate large amounts of re-replication in MCF10a and Sk-Br-3 72 hours post treatment.

By western blotting for NCOAT, OGT, and Emi1, we found that knockdown of these proteins was not efficient, with little to no change observed in the the protein levels of these genes between siRNA treatment and control (GL2) (Fig. 1d). This experiment must be repeated to optimize the transfection conditions in order to achieve efficient co-knockdown. We will start by adjusting the quantity of siRNA oligo introduced as well as titrating the amount of transfection reagent used per sample. Additionally we can modify the order of combination of reagents to allow liposome formation prior to adding the siRNA oligo to the lipofectamine bubbles. In testing the antibodies against Emi1, OGT, and NCOAT, we obtained clean signals on the western blots for Emi1 and OGT, but need to optimize the conditions using the NCOAT antibody.

Task 2 and 3, immunohistochemistry of global O-GlcNAc in cancer samples and developing antibodies to O-GlcNAc modified p53 and ER will be performed pending the result of successful knockdown experiments from Task 1. Meanwhile, we have antibodies that will recognize p53 and the ER as well as antibody that recognizes O-GlcNAc, so immunoprecipitation can be used to determine if O-GlcNAc modifications of these proteins are present under different conditions. Following these experiments, we will examine the effects of induced re-replication on long term genomic stability and malignant transformation. In addition, we will perform these experiments in conjunction with the introduction of DNA damage regulated miRNAs (see below) to enhance cell survival.

Identifying microRNAs induced/repressed by DNA damage that affect cell survival

We are examining the regulation of microRNA expression by DNA damage to identify miRNAs whose expression is changed following DNA damage. This screen is intended to identify miRNAs that play a role in maintaining control of survival, cell cycle regulation and genomic stability following genotoxic stress. We exposed the p53 wild type cell line MCF7 and p53 mutant cell line Sk-Br-3 cell to 5 gray of ionizing radiation(IR) and collected RNA by trizol extraction 24 hours post radiation. The samples were further purified using the Qiagen RNeasy kit and sent to Exiqon to be hybridized to microarrays of complementary oligonucleotides to all currently identified human miRNAs. miRNA expression was compared between the irradiated and control samples for each cell line. In MCF7, only one miRNA, miR642, had a greater than 2 fold difference in expression with miR642 levls being downregulated following irradiation. In Sk-Br-3, the expression of 9 miRNAs was changed more than 2 fold, all of them reduced after exposure to IR (miR-662, miR-492, miR-637, miR-193a, miR-583, miR-939, miR-371, miR-628, and miR-363*). We also chose to study further 4 miRNAs that were each upregulated less than 2 fold on the microarray, miR-34a, miR-29a, miR-29c and miR-21 to confirm that they are indeed induced, if to a lesser extent than expected.

miR29c is upregulated following DNA damage by ionizing radiation

To confirm the results of the microarray, we performed qRT-PCR to measure the expression of each of the identified miRNAs following 5gy IR. Primers of identical sequence to these 14 miRNAs were obtained for use with the Invitrogen NCODE miRNA qRT-PCR kit. qRT-PCR was performed. Consistent with our microarray results, miR-29c(MCF7 p=0.006, Sk-Br-3 p=0.004), miR-29a(MCF7 p=0.02), and miR-21(MCF7 p=0.09) were found to be upregulated following DNA damage (Fig. 2). In contrast, among the miRNAs downregulated on the microarray, miR-642(MCF7 p=0.13, Sk-Br-3 p=0.02) was found to be upregulated and all the others did not demonstrate any significant change after IR. This was somewhat of a disappointment, perhaps due to the fact that we achieved a low yield of detected miRNAs on our arrays, with only around 150 being detectable in each comparison. However, the expression miR-29a and miR-29c were measured to be upregulated to a greater extent by qRT-PCR. This low yield may be due to the lower sensitivity and dynamic

range of detection on this type of microarray. Massively parallel RNA sequencing may be a useful technique to use to improve the yield and threshold of detection in this experiment in the future.

Basal levels of miR-29c are higher in p53 mutant cell line Sk-Br-3

To examine in further detail the induction of our miRNA hits, we performed a time course by treating MCF7 and Sk-Br-3 cells with 5 gy of ionizing radiation and collected samples at 12, 24, and 48 hours post irradiation. RNA was isolated by trizol extraction for northern analysis, and whole cell protein extracts were collected as well for western blotting. By northern blotting, miR-29c was induced in both cell lines after irradiation (Fig. 2d), with a higher basal level in the p53 mutant cell line Sk-Br-3. miR-29c has been reported to affect cell survival by targeting negative regulators of p53, and thus is of interest in the context of response to DNA damage.

Are miR-29c targets downregulated following DNA damage?

To examine potential effects of miR-29c after DNA damage, we used the Miranda algorithm to predict target genes for miR-29c. We obtained primers to several predicted targets of interest and performed qRT-PCR to measure changes in gene expression. None of the potential targets were downregulated at the level of mRNA 24 hours post irradiation (Fig. 3a, 3b). This does not rule them out as targets completely as miRNAs can act at both the level of mRNA stability as well as inhibition of translation. In addition downregulation of these targets may occur 2-3 days after the initial induction of miR-29c, so similar analysis must be performed at later time points.

We western blotted for cyclin E and PTEN, two predicted targets of miR-29c which regulated cell cycle and cell survival, after transfecting MCF7 cells with siRNA mimicking miR-29c, 2'-o-methyl antisense oligo inhibitor of miR-29c, or siRNA against GL2 with and without treatment with 5gy IR. We found no change between the untreated samples, or between the radiation treated samples and their untreated controls (Fig. 4a, 4b).

Other studies have shown that p53 is indirectly upregulated by miR-29c, however when 2'-0-methyl antisense oligo inhibitor against miR-29c was transfected in MCF7 cells, we also found that the p53 response to radiation was unchanged with respect to GL2 (Fig. 4b). However, it remains difficult to quantify the effect of such antisense oligos as levels of the miRNA often are unchanged by northern or RT-PCR while its action is inhibited.

To determine the efficacy of our siRNA mimic to miR-29c(si29c) and 2'-O-methyl antisense inhibitor(2'-O-m 29c), we generated a luciferase reporter construct containing target sequence complementary to miR-29c. This construct was transfected into MCF7 and Sk-Br-3 cells along with combinations of si29c and 2'-O-m 29c (Fig. 4c). The effect of si29c on the reporter was modest, yet co-transfection with 2'-O-m 29c did not rescue this effect. Expression of the luciferase reporter increases with the transfection of 2'-O-methyl 29c

alone in Sk-Br-3 cells. Since these cells have a higher basal miR29c expression, this result is consistent with the inhibition of endogenous but not exogenous miR29c by the 2'-O-methyl antisense. There are several conditions that we will adjust to improve the results of this assay, including quantity of the luciferase reporter, quantity of the normalizing Renilla luciferase plasmid, order of transfection with siRNA and 2'-o-methyl antisense oligo as well as the concentration of oligo.

Transfection of siRNA mimic of miR-29c decreases MCF7 cell survival following DNA damage

MiR-29c is speculated to act in a pro-apoptotic role by the downregulation of MCL1, an anti-apoptotic protein related to BCL-2, as well as by upregulating p53, thus we speculated that its upregulation post-DNA damage could impact long term cell survival, and ectopic expression of miR-29c may impair cell survival following DNA damage. To test this, we transfected si29c in MCF7 cells 48 hours prior to irradiation with doses of 0-5 gy IR. We then assessed survival by colony formation assay and found it to be reduced at several doses, indicating that miR-29c does indeed negatively regulate cell survival following DNA damage (1gy, p=0.01, 2gy p=0.08, 3gy p=0.002) (Fig. 4d).

Basal levels of miR-29c inversely correlate with the presence of p53

We observed that in the p53 mutant cell line Sk-Br-3, miR-29c was expressed at a higher basal level, therefore we examined its expression following depletion of p53 in the wild type cell line MCF7. MCF7 cells were treated with siRNA against p53 or firefly luciferase GL2, and treated with 5gy IR to examine the DNA damage response. As shown, si-p53 results in higher expression of miR-29c, but the induction following DNA damage is no longer observed, indicating there exists a potentially complex p53 dependent regulatory loop controlling miR-29c (Fig. 3c). We also examined expression without and following DNA damage in p53+/+ and p53-/- HCT116 colon cancer cells, and found that basal expression was higher in the absence of p53, while it was induced following radiation in the presence of p53, but in a more transient fashion than MCF7 (Fig. 3d).

Key accomplishments:

- Obtained reagents for quantification of OGT, NCOAT, O-GlcNAc Emi1.
- Generated RNAi oligos for OGT, NCOAT, Emi1
- Performed knockdown experiments in breast cancer and normal mammary epithelial cell lines, determined further troubleshooting needed to generate efficient knockdown and effectively determine effects on cell cycle and genomic stability.
- Identified miRNA's regulated by DNA damage in breast cancer cell lines MCF7 and Sk-Br-3 by microarray
- Confirmed miRNA's upregulated following DNA damage by qRT-PCR and northern blotting
- Examined protein and RNA level of targets of miR-29c following IR induced DNA damage

- Identified miR-29c as having an anti-survival and radiosensitizing effect on breast cancer cells following DNA damage
- Identified a p53 dependent regulation of miR-29c independent of DNA damage

Reportable Outcomes:

Conclusions:

O-GlcNAc and Emi1 in genomic instability of breast cancer.

Preliminary experiments examining Emi1 depletion and perturbation of O-GlcNAc signaling seem to show a modest effect in generating re-replication in MCF10a normal mammary epithelial cells, but we can expect to see greater effects once knockdown efficiency is improved. This can be solved by multiple approaches including optimization of the siRNA transfection conditions, like increasing the oligo concentration and quantity of lipofectamine transfection reagent, use of short hairpin RNA encoding vectors, and chemical inhibition of OGT and NCOAT. There is some indication that inhibition of Emi1 and NCOAT may synergistically generate re-replication in normal breast epithelial cells, which is a positive sign for the future of the project. Once these cell cycle effects can be confirmed, we will examine whether they can lead to sustained aneuploidy, and whether interference in these pathways can lead to malignant transformation in a cell culture model, perhaps along with previously characterized oncogenes, or miRNAs we identify to be responsive to DNA damage. Understanding how interfering with the nutrient sensing mechanisms of cells can affect their genetic stability could lend some insight into the development and survival of cancer within the nutrient conditions of the tumor environment. Understanding the cellular mechanisms cancers use to survive despite a harsh tumor environment could potentially provide new therapeutic targets that are specifically effective in the local physiologic conditions cancer cells exist in.

Determining the role of microRNAs in the response to DNA damage in breast cancer.

Additionally we have identified at least one microRNA that is upregulated in breast cancer following DNA damage, which may function to increase the rate of cell death following genotoxic stress. miR-29c seems to be regulated by p53, which it has already been shown to regulate itself, setting up the potential for a microRNA mediated negative feedback loop tying into one of the most important pathways in cell cycle checkpoint regulation that cancers have to bypass. Elucidating this mechanism of regulation and how it may be perturbed and bypassed could provide clues to the development of breast cancers in different p53 backgrounds, as well as potential targets for the modulation of tumor radiosensitivity. These targets could lead to radiosensitizer therapeutics in the future which could dramatically improve the efficacy of radiation therapy of breast cancer down the road.

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8. Gondi, C.S., Rao, J.S., *Concepts in in vivo siRNA delivery for cancer therapy.* J Cell Physiol, 2009. **220**(2):285-291

Appendices:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Adam Mueller	POSITION TITLE Graduate Student
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harford Community College	AS	1999	Biological Sciences
UMBC	BS	2002	Biological Sciences
UMBC	MS	2003	Applied Molecular Biology
University of Virginia School of Medicine	MD/PhD (ongoing)	2004-Present	Biochemistry and Molecular Genetics/Medicine

Please refer to the application instructions in order to complete sections A, B, and C of the Biographical Sketch.

Research Positions:
1999-2000: Student Research Contractor, Biomaterials, Aberdeen Proving Grounds, Edgewood, Maryland. Study of bacterial enzymatic digestion of V and G type nerve agents. PI: Ilya Elashvili
2002-2003: Graduate Student: Applied Molecular Biology Lab, UMBC, Inducing expression of MHC class II antigen presenting molecules in breast cancer cells. Lab Manager: Julie Wolf, PI: Suzanne Rosenberg.
2004: Rotation Graduate Student, UVA, Michael Weber Lab. MAP kinase signaling in SKOV3 ovarian cancer cells.
2005: Rotation Graduate Student, UVA, Isa Hussaini Lab. PKC signaling in Glioblastoma multiforme.
2005-Present: Graduate Student, UVA, Anindya Dutta Lab. Effect of statins on progression of breast cancer, as well as screening of growth essential genes in normal breast tissue.

Awards and Honors:
1996: HCC Alfred C' O'Connell Scholarship
1999: HCC Academic Achievement Award
1999: Maryland Burgee Board of Regents Scholarship
2004: University of Virginia Graduate School of Arts and Sciences Fellowship
2005: NIH Cancer Training Grant
Member Phi Theta Kappa Honors Society (Treasurer Rho Beta 1998)
Member Golden Key Honors Society
Member Phi Kapp Phi Honors Society

Non-Research Positions and Activities:
2000-2001: Assistant Coach. Joppatowne Tigersharks Swim Team.
2003: Associate Game Designer/Web Administrator. GRS Games/Gentle Revolution Press. Supervison: Victoria Kinnear
2002: President, 2003-2004 Assitant Instructor. UMBC Jujitsu Club.
2006-2007. Instructor, UVA Brazilian Jiu-Jitsu club.

Supporting Materials:

Cell line	miRNA	Fold Downregulated after IR
MCF7	miR-642	2.82
Sk-Br-3	miR-665	5.82
	miR-492	4.97
	miR-637	4.83
	miR-193a-5p	4.09
	miR-583	2.19
	miR-939	2.15
	miR-371	2.14
	miR-628-3p	2.06
	miR363*	2.02

Table 1. miRNAs downregulated more than 2 fold 24 hrs. following 5gy IR.

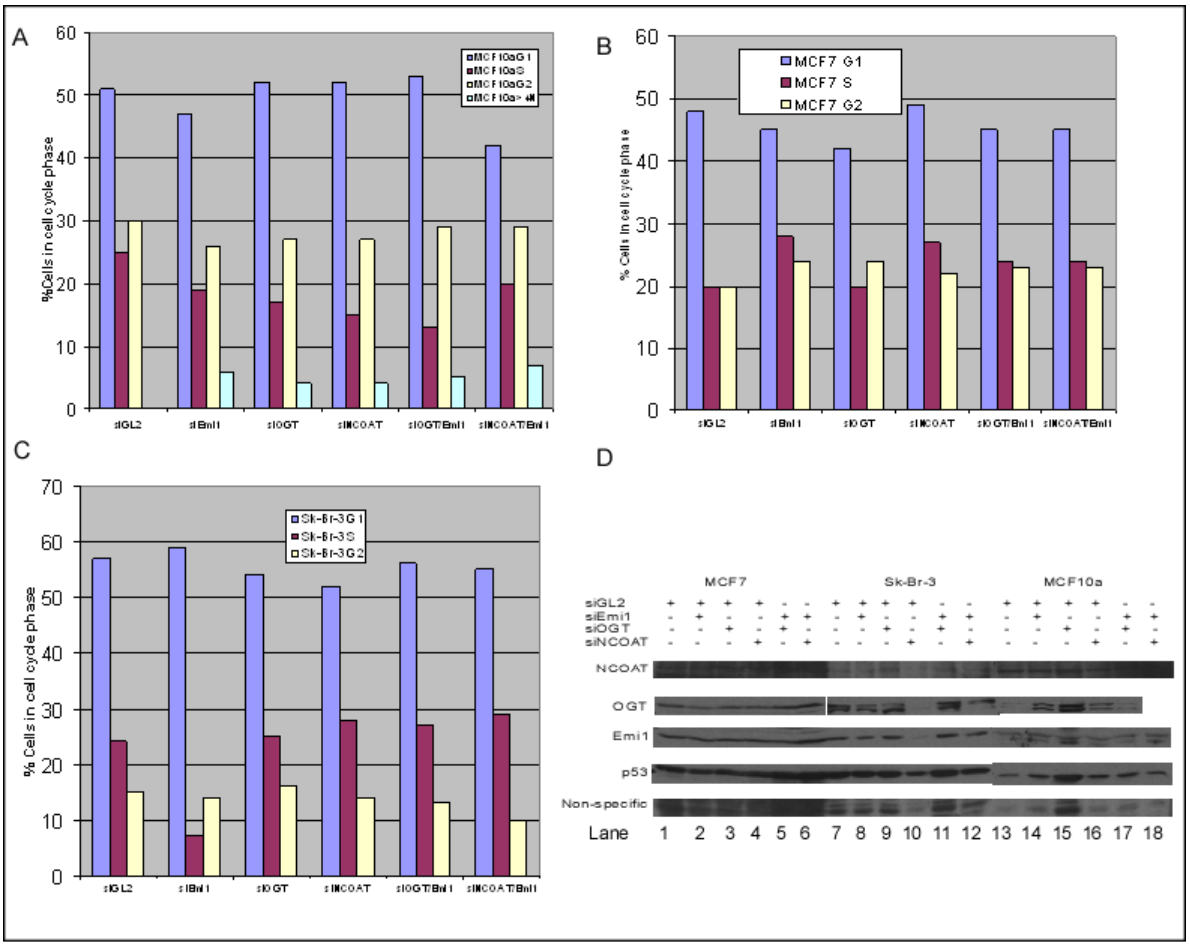

Figure 1. siRNA of Emi1, OGT, NCOAT has little effect on cell cycle of MCF10a(a), MCF7(b), MCF7(c). This appears due to inefficient knockdown of Emi1, OGT, NCOAT, non-specific band shows under-loading in lane 10 and overloading in lane 15 (d). Optimization of knockdown conditions should rectify this.

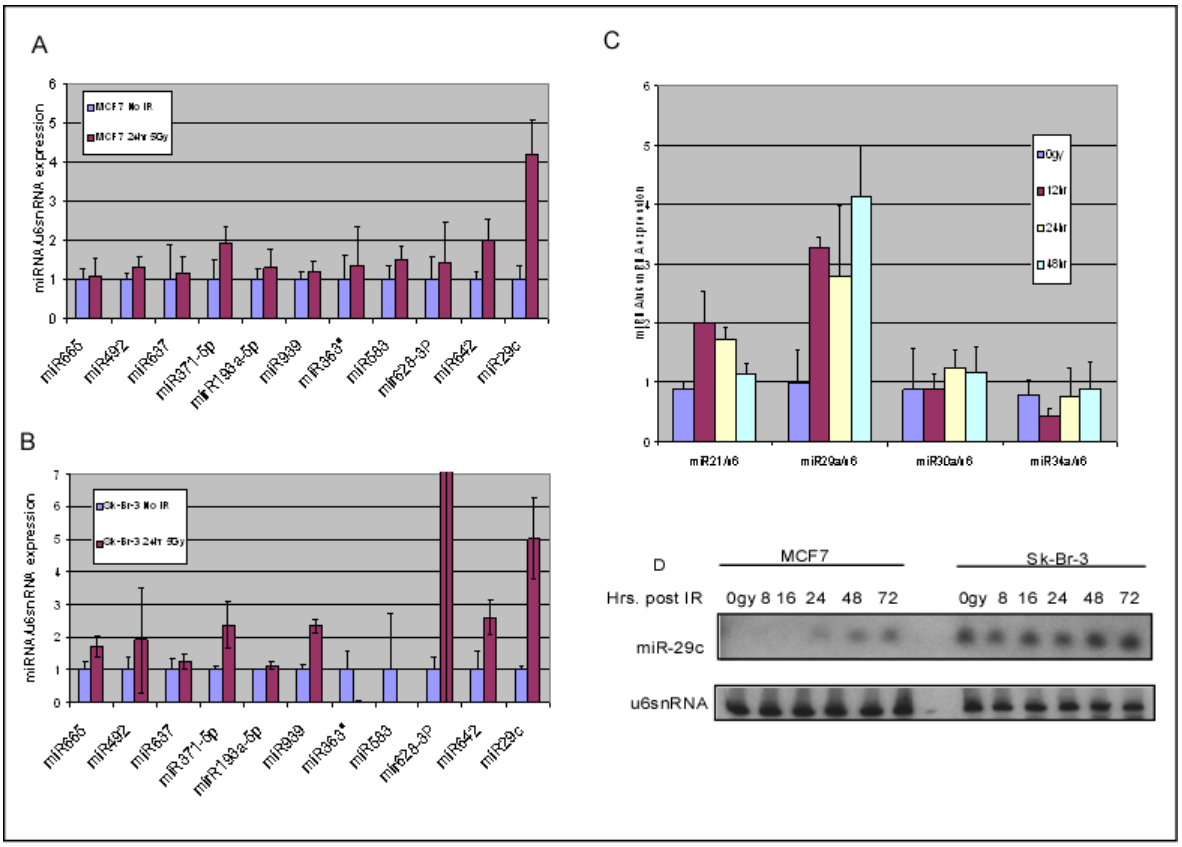

Figure 2. miR-29c and 642 are upregulated following 5gy ionizing radiation in MCF7(a) and Sk-Br-3(b) breast cancer cell lines. In a 48 hour time course, miR29a and miR21 show sustained upregulation following DNA damage(c). By northern blot miR-29c is upregulated in MCF7 and Sk-Br-3, but is at a higher basal level in the p53 mutant Sk-Br-3 cell line(d).

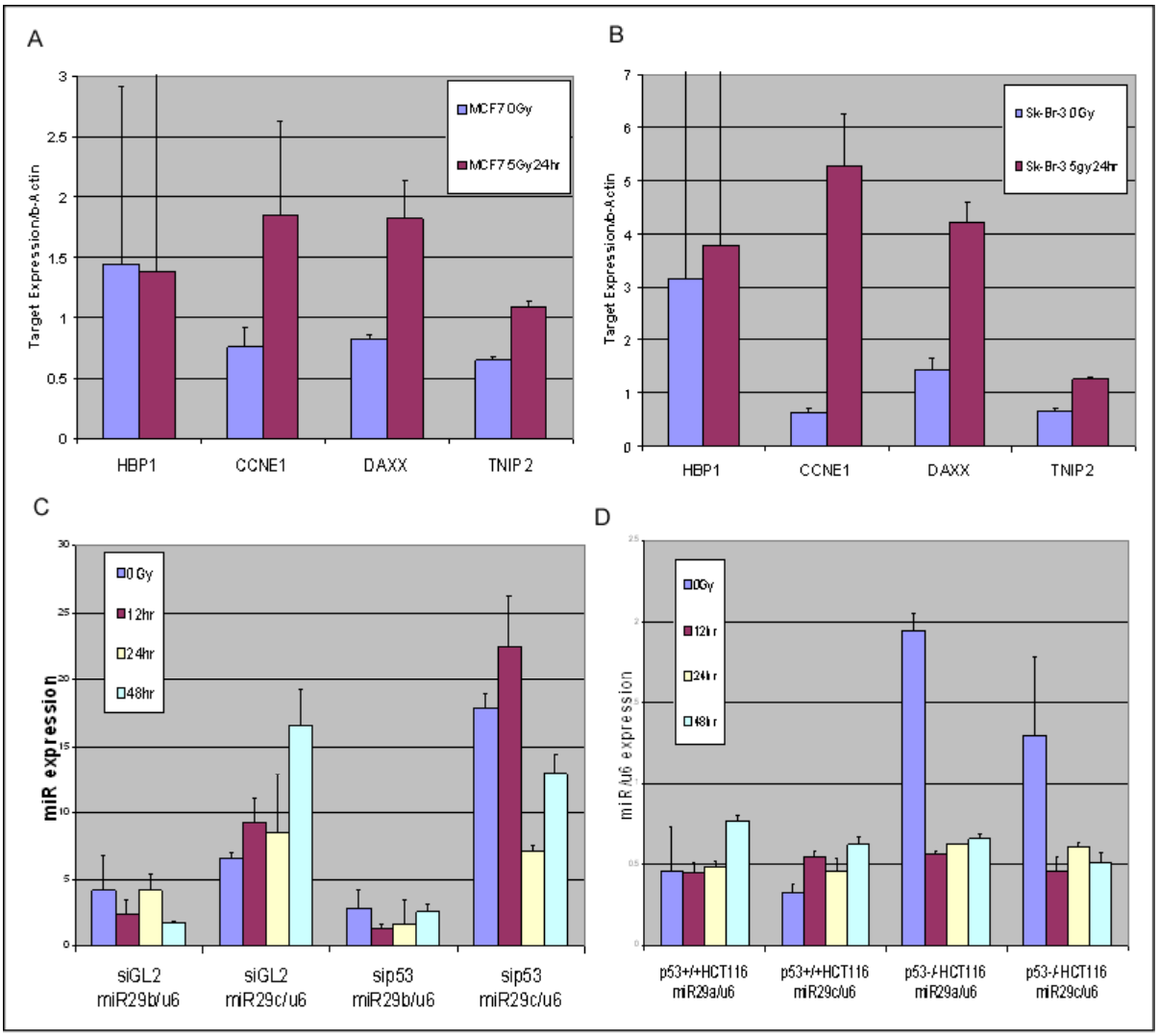

Figure 3. Targets of miR-29c are not downregulated at the mRNA level 24 hours following DNA damage in MCF7 and Sk-Br-3(a-b). siRNA of p53 results in a higher basal level of miR-29c expression in MCF7(c). miR-29c has a higher basal expression in p53-/- HCT116 cells than p53+/+ HCT116(d).

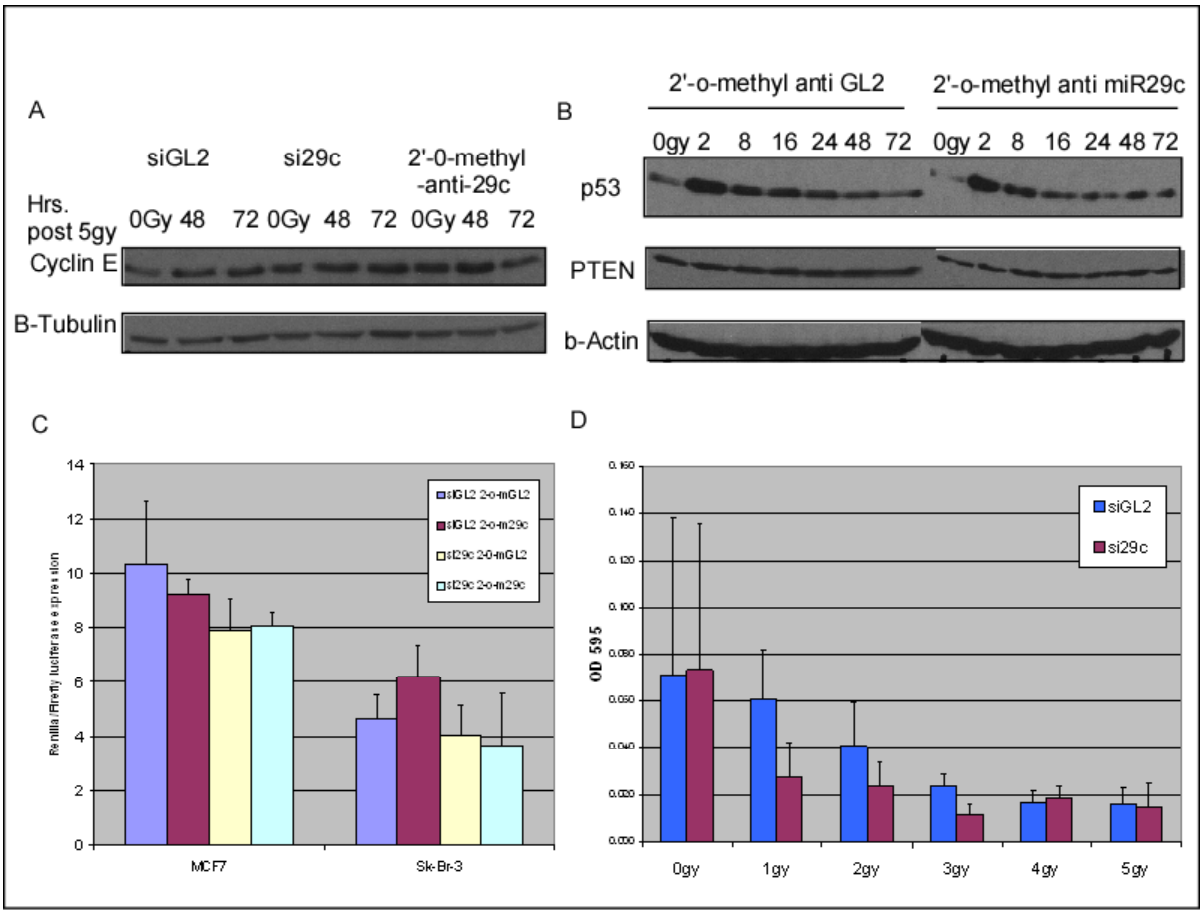

Figure 4. siRNA mimic of miR29c, and 2'-0-methyl antisense 29c oligos have no effect on cyclinE expression following IR(a). 2'-0-methyl antisense oligo blocking of 29c has indeterminate effect upon PTEN and p53 post IR(b). 2'-0-methyl antisense oligo to miR29c not rescuing luciferase expression in the presence of si29c, si29c has only modest effect on downregulating reporter containing its target sequence(c). Introduction of si29c has a negative effect on the survival of MCF7 cells by colony formation assay following IR(d).